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Molecular pathogenesis of alcohol withdrawal seizures: the modified lipid–protein interaction mechanism

C.Y.K. TAN* & D.F. WEAVER*†

**Department of Chemistry and †Department of Medicine, Division of Neurology, Queen's University, Kingston, Ontario, Canada K7L 3N6*

Correspondence to: Dr D.F. Weaver, Department of Chemistry, Queen's University, Kingston, Ontario, Canada, K7L 3N6

The phrase alcohol withdrawal seizures (AWS) refers to seizures that result from the withdrawal of alcohol after a period of chronic alcohol administration. A mechanism of AWS is postulated, namely the modified lipid–protein interaction (MLPI) mechanism. This hypothesis is based upon an evaluation of the mechanisms of membrane fluidity, calcium channels, γ-aminobutyric acid (GABA) and glutamate in the molecular pathogenesis of AWS. The mechanism hypothesizes that acute ethanol treatment alters the neuronal membrane lipids which then perturbs protein events, such as affecting the $GABA_A$ receptors, NMDA receptors and voltage-dependent Ca^{2+} channels synergistically or in combination. Subsequent adaptations in these systems occur after prolonged administration of ethanol. A sudden withdrawal of ethanol then leads to hyperexcitability which results in AWS.

Key words: AWS; MLPI; membrane fluidity; cell calcium; GABA receptors; NMDA receptors.

INTRODUCTION

Ethanol is one of the oldest pharmacological agents. Although evidence of alcohol use dates to the Stone Age, alcohol-induced biological effects constitute the oldest of pharmacological puzzles[1]. The relationship between alcohol use and seizures is well known. For many years, alcohol was considered a toxic substance that precipitated seizures[2]. This conclusion evolved from observations that seizures occur in people using alcohol in excess. Studies indicated that epilepsy occurred in 25% of alcoholic individuals, and that about 15% of epileptic patients were 'abnormal drinkers'[2]. These results showed that the frequency of alcoholism and seizures or seizures occurring with heavy alcohol drinking far exceeded that which might have been expected by chance alone. More recently, the phenomenon of alcohol withdrawal seizures (AWS) has been recognized as a relevant clinical issue.

Alcohol withdrawal seizures are a seizure state related not simply to the abuse of alcohol but specifically to the cessation of drinking (withdrawal) following a period of chronic intoxication[3]. The symptoms of withdrawal manifest themselves after the period of intoxication has subsided for 6–7 hours. In addition to tremulousness, three major groups of withdrawal symptoms were reported by Victor[3]; (1) a state characterized mainly by tremor and hallucinations, which peaks in intensity 24 hours after cessation of drinking; (2) convulsive seizures that occurred singly or in short bursts, in most cases between 7 and 48 hours after withdrawal, with a peak incidence at 24 hours (different peak times have been reported[4]); and (3) a state characterized by gross tremor and agitation, disorders of sense perception, and increased psychomotor and autonomic nervous system activity, known as delirium tremens.

Alcohol withdrawal symptoms have some characteristics of generalized tonic–clonic seizures[3], but there is no neocortical seizure focus, and the EEG during sober periods is normal[5]. In addition, Tabakoff and Rothstein[5] reported that approximately half of patients with AWS demonstrated myoclonus or convulsive seizures following photic stimulation. Although AWS can therefore be distinguished from epileptic seizures, they are more likely to occur in epileptics, since alcohol withdrawal lowers the seizure threshold[5].

Many hypotheses have been proposed to explain the mechanism(s) responsible for AWS, but these have not yet been proved. The conventional hypothesis has been that ethanol is a non-specific drug that acts by

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perturbing neuronal membranes[6,7]. More recent data, however, have shown that ethanol specifically and selectively affects the function of certain membrane-bound proteins, and ion channels. Among the proteins are glutamate (Glu) receptor-gated ion channels (particularly *N*-methyl-D-aspartate (NMDA)) and γ-aminobutyric acid (GABA) receptor-gated ion channels. The acute effects include inhibition of NMDA-activated ion currents[8], potentiation of $GABA_A$-activated Cl^- current[9], and blockade of Ca^{2+} uptake[10,16] in neurons. Adaptations in these systems occur after prolonged administration of ethanol and have been reported to cause an increased number of NMDA receptors[4,11], reduced $GABA_A$ receptor functions[12,13] and increased numbers of L-type Ca^{2+} channels[14,15]. Consequently, a withdrawal of ethanol then leads to hyperexcitatory states and ultimately causes AWS.

This review evaluates the mechanisms of membrane fluidity, calcium channels, GABA and glutamate in the molecular pathogenesis of AWS. Based upon this evaluation, the modified lipid–protein interaction (MLPI) mechanism is postulated. This mechanism hypothesizes that acute ethanol treatment alters the neuronal membrane lipids which then perturbs protein events. This perturbation then affects $GABA_A$ receptors, NMDA receptors and voltage-dependent Ca^{2+} channels synergistically or in combination. Subsequent adaptations in these systems occur to counter balance the offset of these protein receptor systems after prolonged administration of ethanol. A sudden withdrawal of ethanol then disturbs these systems again, leading to hyperexcitatory and resulting in AWS.

In this review, the definition for lipid–protein interaction is to refer to an association between lipid and protein which when disrupted changes the function of the protein. There are various possibilities for lipid interacting with protein to affect protein function. (1) Thickness of the lipid bilayers alters as the lipid composition of the host membrane changes, which leads to the modification of protein function. Modification of the thickness of the lipid bilayer has been discussed by Johannson *et al*[17], Mouritsen and Bloom[18] and Sperotto and Mouritsen[19]. (2) Alteration of the membrane lipid composition changes the 'fluidity' of the membrane.

EFFECTS OF ETHANOL ON MEMBRANE FLUIDITY

Membrane physical properties

Mammalian cell membranes consist of a lipid bilayer containing a variety of proteins. The lipids are quite diverse, including phospholipids, galactolipids, and neutral lipids[20]. These lipids are not randomly distributed in the bilayers; certain lipids are localized predominantly in the outer leaflet of the bilayer (e.g. gangliosides) whilst others are mainly in the inner leaflet (e.g. phosphatidylserine)[20].

The main physical properties studied in alcohol research are lipid order and viscosity. Order is the measure of packing of the lipids or, more qualitatively, the volume occupied by each lipid molecule. Viscosity is the resistance to the solvent drag or a measure of the freedom of lateral movement of the lipid molecules. The frequently used term 'fluidity' does not have a precise definition but is usually taken to include both order and viscosity[21]. Other membrane characteristics of potential importance are membrane surface charge and lipid–protein interactions, but little is known about the effects of ethanol on these properties.

Ethanol-induced disorder

The most frequent methods used to estimate membrane fluidity include electron paramagnetic resonance (EPR), fluorescence polarization and nuclear magnetic resonance (NMR). Both EPR and fluorescence polarization techniques are similar but sometimes give qualitatively different results, since different probes measure different regions of the membrane that have distinct physical properties and responses to ethanol. Ethanol, and other anaesthetic agents allow exogenous probes (spin labels or fluorescence dyes) to move more rapidly or with greater amplitude than in their absence[22].

In 1977, Chin and Goldstein[23] demonstrated that brain membranes were disordered by *in vitro* exposure to concentrations of ethanol that can be attained *in vivo* (10–20 mM). These investigators used EPR probes inserted into synaptic membranes to study the effects of ethanol. During the next few years, a number of studies[24] using EPR and fluorescence probes demonstrated that ethanol concentrations in the range of 10–100 mM can disorder brain membranes. However, the effect of ethanol was smaller than those caused by altering body temperature a few degrees, and questions arose immediately. Some issues of concern included whether the changes in membrane properties were functionally important and whether the differences in alcohol sensitivity *in vivo* were reflected by differences in membrane sensitivity *in vitro*. To address this question, attempts were made to correlate the membrane fluidity with some measure of ethanol action *in vitro*, as an indication of whether small changes were important. Membranes from mice with genetic differences in alcohol sensi-

tivity and membranes from ethanol-tolerant mice (resulting from chronic consumption of ethanol) were tested. Indeed, sensitivity to the membrane fluidity in the presence of ethanol did correlate well with ethanol sensitivity *in vivo*[23,25,26]. However, the decreased ethanol sensitivity observed *in vivo* during early development and the increased sensitivity that occurred during ageing were not reflected by the changes in membrane sensitivity to ethanol[27]. This implied that some, but not all, alterations in ethanol sensitivity *in vivo* could be correlated with differences in membrane properties.

The fact that the genetic differences in alcohol sensitivity have only been detected at the membrane surface[30] raises the question of where in the membrane is the most pronounced (and presumably most important) action of ethanol. Data from both EPR and fluorescence probes showed that the fluidizing action of ethanol was most pronounced in the membrane core (lower methylene groups of the acyl chains), leading to the conclusion that the action of ethanol becomes stronger toward the interior of the bilayer[30]. This result does not appear to be consistent with the fact that ethanol is not very lipid-soluble, and therefore the highest concentration will be at the membrane surface. However, more recent data from NMR studies supported the alternative hypothesis that the fluidizing action of ethanol was most pronounced at the membrane surface, and that ethanol was located near the membrane surface with the hydroxyl group interacting with the polar headgroup of the phospholipids and with the ethyl group embedded in the membrane[31]. Thus, ethanol may have two distinct actions on the membrane and the EPR and fluorescence probe experiments measure an average of these two actions[20]. NMR may be particularly useful in studying these two sites of ethanol action because NMR does not require the use of an exogenous probe, but measures resonances from the lipids themselves[32,33]. This result also serves as an example that different methods of measuring membrane fluidity may lead to different interpretations regarding the actions of ethanol.

Recently, Iqbal *et al*[49] showed that ethanol inhibited [^{125}I]calmodulin (CaM) binding to synaptic plasma membranes from rat brain and decreased 1,6-dephenyl-1,3,5-hexatriene (DiPH) fluorescence polarization (i.e. increased membrane disorder) in the concentration range of 25–300 mM. This inhibition was correlated in a concentration-dependent manner with the increased membrane fluidity. In this concentration range, the inhibition of [^{125}I]CaM binding was 17% at 25 mM, 45% at 100 mM and 68% at 300 mM. The decreased DiPH fluorescence polarization was 0.0022 at 25 mM and 0.0182 at 300 mM. These are in good agreement with those reported in earlier studies using similar DiPH fluorescence polarization[26,29] or EPR spectrometry[25,28], that showed a correlation between ethanol and membrane fluidity.

Adaptation and tolerance in membrane

One might expect the adaptation of the membrane after chronic treatment with ethanol would result in the 'too-fluid' membrane pulling itself together and adjusting its properties to compensate for the continuous presence of ethanol (adaptation). Indeed, there are many examples of resistance to the fluidizing effect after chronic administration of ethanol[23] and of decreasing membrane fluidity in the absence of ethanol[28,29]. However, the effect is not universal; it depends on the tissue, on the method of examining the membranes, and perhaps on the conditions of ethanol administration. For example, the order parameter of mouse synaptosomal plasma membranes from ethanol-treated mice is higher than that of controls when the spin label is 12-deoxylstearic acid but not when it is 5-deoxylstearic acid[23,28].

Whether or not the membranes of ethanol-treated animals are more ordered than normal, they are usually resistant to ethanol-induced disordering *in vitro* (tolerance). In a study by Goldstein[34], tolerance developed in mice over a few days of ethanol exposure, and it decayed rapidly within 30 hours of withdrawal. The magnitude of the tolerance was about twofold or in other words, the tolerant mice can exhibit functional balance at a brain concentration of ethanol nearly twice that of controls[34].

Rottenberg and co-workers[35,36] demonstrated a decreased solubility of ethanol in membranes from ethanol-treated rats, and they suggested that the membrane tolerance was simply the result of lower concentrations of ethanol within the membranes of the ethanol-treated animals. This might indeed explain the tolerance due to the reduced solubility of ethanol when the membranes are rigid.

Chemical basis for increased order

The experiments described above demonstrated that membrane tolerance and increased order after chronic ethanol administration are frequent but not universal findings. To discover the causes of membrane stiffening after chronic ethanol treatment, investigations on the chemical changes in membranes have been performed. The search for altered lipids focuses on cholesterol and saturated acyl chains of phospholipids because increased amounts of these components are expected to stiffen membranes and to render them relatively resistant to ethanol-induced disorder. Indeed, in some situations, the membrane choles-

terol/phospholipid ratio does increase during ethanol treatment, for example in mouse synaptosomal membranes after treatment of mice with ethanol by diet[37].

Cholesterol does not always increase after chronic ethanol treatment. In some mice studies, when tolerance and physical dependence was developed, membranes showed corresponding cholesterol increases[28]. But in other similar experiments, it was found that there was no change in the cholesterol/phospholipid ratio of the brain synaptosomal plasma membranes[38]. Others have even found a decreased cholesterol/phospholipid ratio in synaptosomal membranes from ethanol-treated animals[29].

Membranes from ethanol-treated animals often showed an apparently analogous increased saturation of acyl chains[38,39], but this again was not a universal finding. Sometimes the membrane fatty acids became more unsaturated, contrary to prediction[40,41]. Several investigators, including Smith *et al*[42], commented on the possible confounding effects of dietary and nutritional factors on these different responses to ethanol. Studies showed that, in some cases, ethanol enhanced the depletion of polyunsaturated fatty acids in the brain when the diet was deficient in these fatty acids[43], whereas others found no changes among the polyunsaturated fatty acids[44,45].

Thus, there is currently no simple model to demonstrate the role between saturated and polyunsaturated fatty acids which is explained by or correlated with the compensatory reorganization of neuronal membranes that have adapted to ethanol. In addition, adaptations may proceed by various mechanisms simultaneously[22]. If the primary objective of the adaptation is to counteract disorder, the organism may use cholesterol, fatty acid chains, and other mechanisms simultaneously, and one or another may be detectable to the investigator after different periods of ethanol exposure[22].

Recently, a pathway for alcohol metabolism which yields an unusual phospholipid phosphatidylethanol (PEt) product had been reported[46], Omodeo-Sale *et al*[47] reported that at higher PEt concentrations (5–10% of the total phospholipids), PEt induced an increase of fluidity of artificial and natural bilayers and that it was able to confer membrane tolerance to the fluidizing effects of ethanol. However, at physiological PEt concentrations (1–2% of total phospholipids), there seems to be little effect on membrane fluidity or on the tolerance to ethanol. On the other hand, one cannot exclude that in some membrane microenvironments these effects might be amplified by a local increase of PEt concentration.

It is speculated that by hydrophobic head group of PEt might perturb the membrane architecture by disrupting the hydrogen or ionic bond lattice extending over the surface of the membrane[47]. For example, Omodeo-Sale[47] found that incorporation of small amounts of PEt (2% of the total phospholipids) resulted in significant changes in the activity of Na^+/K^+ ATPase and 5′-nucleotidase, reducing the Na^+/K^+ ATPase activation and enhancing 5′-nucleotidase activity. These authors suggested the PEt-induced reduction of Na^+/K^+ ATPase activity could be related either to the resistance to ethanol-induced fluidization acquired by membranes enriched with PEt, or to a more specific influence of PEt on the catalytic site of the enzyme, or on some lipid–lipid or lipid–protein interactions. The PEt-induced increased 5′-nucleotidase activity could be caused by a direct interaction of the polar head group of PEt with the catalytic portion of the enzyme at the membrane surface where it was located[48].

Summary of ethanol–membrane interactions

Although the adaptive lipid modifications brought about as a result of chronic ethanol treatment remains an attractive hypothesis, testing has revealed many inconsistencies. One may find a large body of observations that fit the general outlines of the idea, but there are many exceptions. Perhaps these will provide clues to the actual mechanism of responses to ethanol.

Even though there is a general consensus about the effects of ethanol on membrane fluidity, the key question is whether any of these effects are of physiological importance. Investigators have pointed out that the observed changes may be too small (1% change in the order parameter) to alter membrane function. Nevertheless, the membrane fluidity hypothesis has yet to be disproved. Alternative hypotheses can also explain the correlation between ethanol and membrane fluidity changes. For example, ethanol could act on specific protein sites which are within the membrane; thus, ethanol action would be proportional to the membrane concentration, which, in turn, is proportional to the change in membrane fluidity[20]. Alternatively, the action of ethanol may be at another site (e.g. membrane calcium binding). Moreover, it can be due to the unusual phospholipid(s) produced by alcohol metabolism (e.g. PEt) that exert their effects on the physicochemical properties of the membrane or perhaps on the proteins themselves.

The interaction of ethanol with neuronal membranes, including lipid and protein components, causes significant changes in membrane function. Furthermore, it has not been proven that a single effect, such as an alteration in membrane fluidity, is solely responsible for all functional alterations. The studies of cellular calcium, NMDA receptors and GABA receptors (discussed below), sug-

gests AWS may be due to combination effects on calcium channels, NMDA receptors and GABA receptors, which results from the alteration(s) in neuronal membrane lipids during chronic ethanol treatment; it is likely that these effects cause AWS as the ethanol is withdrawn. Thus, the proposed mechanism is that acute ethanol treatment alters the neuronal membrane lipids; this alteration then perturbs protein events that in turn affect the $GABA_A$ receptors, NMDA receptors and voltage-dependent Ca channels synergistically or in combination. Subsequent adaptations in these systems occur after prolonged administration of ethanol, and a sudden withdrawal of ethanol then causes hyperexcitation which results in AWS.

EFFECTS OF ETHANOL ON CELL CALCIUM

Ethanol is known to alter both intracellular Ca^{2+} concentrations and Ca^{2+} movement across cell membranes[50]. In addition to passage through ion selective channels, controlled by transmembrane potential and ion concentration gradients, Ca^{2+} is actively moved via membrane exchange with sodium and by Ca^{2+}-activated ATPases. It is the influx of Ca^{2+} which can lead to seizure activity and cell death. Ion channels permeable to Ca^{2+} include both voltage-gated and ligand-gated processes. Voltage-operated Ca^{2+} channels have been divided into subtypes, according to their voltage dependence, inactivation characteristics, and responses to drugs[50].

Electrophysiologic evidence[51] suggested that at least four distinct types of voltage-operated Ca^{2+} channels are present on neurons. They have different pharmacological and electrical properties. Channels with long opening periods which need considerable depolarization for activation, but inactivate slowly, have been designed L-type. Channels that open transiently (T-type) require low levels of depolarization for activation but inactivate very rapidly. Those with an intermediate opening period (N-type) require considerable levels of depolarization for activation but inactivate rapidly. The Ca^{2+} channel types are distributed regionally: those on the terminals being largely the N-type, whereas those on cell bodies and dendrites appear to be of the L- and T-types[69].

Only the L-type channel is susceptible to inhibition by clinically available Ca^{2+} channel antagonists. The most potent group of Ca^{2+} channel antagonist are the 1,4-dihydropyridines (DHPs); the L-type channel is sometimes described as the DHP-sensitive channel. However, DHPs may not be as selective for the L-type channel in normal mammalian neurons as for those in cultured neurons. For example, in hypothalamic cells, DHPs are found to affect channels that possess the voltage dependence and inactivation characteristics of L-type channels and those with characteristics of the T-type channels[52]. In addition, DHPs can have either antagonistic effects or agonistic effects on the channel[53]. Therefore, one cannot simply assume that the functional consequences of a DHP antagonist will always reflect inhibition of depolarization-induced Ca^{2+} entry. Acute ethanol administration and chronic ethanol intake have been reported to alter intracellular Ca^{2+} concentrations, Ca^{2+} uptake, and Ca^{2+} currents[50].

Acute effects of ethanol on cell calcium

Acute effects on intracellular calcium concentrations. Harris[54] found that ethanol (50 mM–1.6 M) decreased ATP-dependent Ca^{2+} uptake by lysed synaptosomal tissue. Similar results were obtained after acute administration of ethanol (4 g/kg) *in vivo*. Chronic treatment with ethanol conferred tolerance to its effect on ATP-dependent Ca^{2+} uptake, and decreased ATP-dependent uptake in the absence of ethanol[54,55]. Shah and Pant[56] showed that ethanol (30–500 mM) induced Ca^{2+} release from microsomes, prepared from purified synaptosomes. Consequently, intracellular Ca^{2+} concentration was increased by the acute effect of ethanol.

The concentrations of ethanol that affected intracellular Ca^{2+} levels were considerably higher than those achieved during the behavioural actions of social ethanol ingestion. This suggests that alterations in intracellular Ca^{2+} concentration might not play a significant role in the mechanism(s) of the acute actions of ethanol. However, in the majority of the studies described above, measurements were made using synaptosomes. Synaptosomal Ca^{2+} concentrations do not necessarily reflect those in other regions of the neuron. It is becoming increasingly apparent that local variations in Ca^{2+} distribution occur in different areas of the cell, and these may play an important role in the control of excitability[50]. The possibility remains, therefore, that local changes in the intracellular Ca^{2+} concentration may be important in the mechanism of action of ethanol.

Acute effects of ethanol on calcium uptake. Evidence on the effects of ethanol on Ca^{2+} uptake into neurons has been conflicting. For example, Blaustein *et al*[57] found no change in synaptosomal uptake on addition of 100 mM ethanol, while Harris and Hood[13] reported that 50–800 mM ethanol decreased K^+-stimulated Ca^{2+} uptake in brain synaptosomes, but the changes were small until very high concentrations were reached. In contrast, Friedman *et al*[58] found that ethanol increased Ca^{2+} influx into mouse whole-brain synaptosomes at 80 mM. A similar effect was seen

45 minutes after administration of an acute ethanol dose (4.5 g/kg) *in vivo*. On the other hand, Stokes and Harris[59] found that ethanol inhibited Ca^{2+} uptake into synaptosomes, with greater action on the cerebellum and striatum than on cerebral cortex and brain and stem.

The fast component of Ca^{2+} uptake into synaptosomes was blocked by concentrations that were associated with the *in vivo* actions of ethanol, but the slow component of uptake was not affected strongly in this range[50]. Leslie *et al*[64] demonstrated an inhibitory action on the slow component at 25 mM. Effects were found on the fast component of Ca^{2+} uptake over longer incubation periods in concentrations out of the relevant range[64]. As a result, the effects of ethanol on Ca^{2+} uptake are inconsistent. Hence, the acute ethanol effects on Ca^{2+} uptake are still questionable.

Acute effects of ethanol on calcium currents. There is considerable evidence for a selective action of ethanol on Ca^{2+} currents. Pozos and Oakes[60] found that ethanol (11–110 mM) decreased the duration of somatic action potentials, which was interpreted as a decrease in Ca^{2+} conductance. At concentrations lower than 11 mM, ethanol increased spike duration, an effect suggested to be due to increased Ca^{2+} conductance and to be related to the hyperexcitability caused by low doses of ethanol *in vivo*. This gives rise to the question of what causes a dependency of Ca^{2+} current on ethanol concentration.

Ethanol at 5–20 mM, as shown by Carlen *et al*[61], can increase the postsynaptic Ca^{2+}-mediated K^+ conductance that underlies after-hyperpolarization in CA1 cells of the isolated rat hippocampus. These authors suggested that the changes were due to either an increased sensitivity to Ca^{2+} or an increase in intracellular Ca^{2+} concentration. Increased sensitivity to Ca^{2+} was also suggested as an important mechanism of action of ethanol by Lynch and Littleton[62], who studied the effect of chronic ethanol on neurotransmitter release. In contrast to the above results, Siggins *et al*[63], also using isolated rat hippocampal slices, found no evidence of such potentiation. Ca^{2+}-activated K^+ currents were either unchanged or decreased by the acute administration of ethanol (11–150 mM). The authors suggested that the differences between their results and those of Carlen *et al*[61] may be due to the method of ethanol administration, because Siggins *et al*[63] used bath perfusion whereas the earlier study added ethanol by microdrop. Benson *et al*[64], who measured the effects of ethanol (50–100 mM) in a voltage-clamp study on hippocampal pyramidal cells, also failed to find an effect on the Ca^{2+}-dependent K^+ current.

Thus the results are conflicting, as with membrane fluidity, but again the differences may be due to the tissues used, the method of examining the membranes, and perhaps the conditions of ethanol administration. Unfortunately, this complicates the determination of the effects of ethanol further.

Effects of chronic ethanol treatment on cell calcium

Daniell and Harris[65] reported that in the absence of ethanol, intracellular Ca^{2+} concentrations were not significantly altered after 7 days on a liquid diet, but the mean value was altered after ethanol treatment. In addition, Friedman *et al*[58] found that the action of ethanol was considerably reduced in tissues from mice treated with a liquid ethanol diet for 10 days (17 g/kg/day), due to tolerance. In the absence of ethanol added to the medium, synaptosomes from animals receiving the chronic ethanol diet showed decreased Ca^{2+} accumulation in both depolarizing and non-depolarizing conditions.

Cultured cells of neural origin (i.e. PC12 cells) showed decreased Ca^{2+} uptake when 50 mM ethanol was added acutely to the medium[66]. When the cells were grown in 200 mM ethanol for 6 days, this action of ethanol was unaltered, but Ca^{2+} uptake in the absence of ethanol was increased by 75%. Greenberg *et al*[67] showed that this increase could be blocked by the dihydropyridine nifedipine, at nanomolar concentrations, suggesting that it occurred through voltage-sensitive channels. Skattebol and Rabin[68], however, found an increase in the effects of ethanol on voltage-dependent Ca^{2+} uptake when PC12 cells were grown in 150 mM ethanol for 4 days. The difference in these studies was suggested by the latter authors to be due to the use of difference subclones of cells. These authors also made the point that the difference between the effects of chronic administration of ethanol *in vivo* and its action on PC12 cells, was likely due to the wide variety of effects of ethanol *in vivo*, in contrast to its direct action on cultured cells[68].

Effects of ethanol on dihydropyridine-sensitive calcium channels

Changes in DHP binding. Greenberg and Cooper[70], Harris *et al*[71] and Rius *et al*[72] found that the acute addition of ethanol *in vitro* had little effect on DHP binding in brain homogenates, except at extremely high concentrations. It was concluded that a direct action on DHP binding was unlikely to be involved in the actions of ethanol (acute treatment). However, this may not be the case in terms of the effects of the membrane potential on DHP binding. DHP Ca^{2+} channel antagonists bind preferentially to the inacti-

vated form of the channel complex[73]. Rius *et al*[72] showed that a single dose (3 g/kg, i.p.) of ethanol, caused an increase in DHP binding of short duration, followed by an increase in affinity with maximum affinity 8 hours after ethanol administration.

Chronic ethanol treatment increased the number of DHP binding sites in the cerebral cortex[17,18], in whole brain[74], and in peripheral excitable tissues[75]. This effect has been reported for a variety of ethanol-treatment schedules: inhalation of ethanol by rats (10, rising to 20 g/kg/day) for 7 days[76,77], i.p. injection of ethanol in rats at 2 g/kg, once daily, for 10 days[18], and administration of ethanol in the drinking fluid to mice (11–14 g/kg/day) for 12 weeks[17]. The inhalation and drinking-fluid methods of administration caused tolerance to ethanol and a mild withdrawal syndrome. The i.p. injection schedule caused tolerance, but no withdrawal signs. The increase in the number of DHP binding sites was variable, depending on ethanol concentration and the duration exposure, but could be well in excess of 100%.

An increase in the number of DHP sites was also found by Wu *et al*[78] who demonstrated a temporal correlation between the increase in binding sites and ethanol tolerance. In addition, an increase in DHP binding-site number has also been found in PC12 cells of a cultured neural cell line grown in 200 mM of ethanol for 6 days[66]. The increase in DHP binding sites was prevented by the protein synthesis inhibitor cycloheximide (at the mRNA translation stage) and by lomofungin and anisomycin (which inhibit mRNA synthesis), suggesting that it was a consequence of synthesis of new channel proteins[79]. As mentioned before, it is unlikely that ethanol acts directly on DHP binding sites, so the question becomes what causes the increase of DHP binding sites after chronic ethanol treatment? In addition, a change in the number of DHP binding sites is of interest only if the increased binding sites represent functional entities. The *in vitro* effects of the DHP Ca^{2+} agonist, Bay K-8644, on neurotransmitter release and phosphotidyl inositol turnover were increased in cortical tissue from rats treated chronically with ethanol by inhalation for 7 days[18]. These authors suggested that the increase in DHP binding sites appeared to reflect an increase in voltage-sensitive Ca^{2+} channels, which implies that the increase in DHP binding sites does represent functional entities.

Recently, Huang and McArdle[80] also showed with their electrophysiological study, that increased Ca^{2+} current from hippocampal neurons of ethanol-tolerant mice was due to an increase in the number of functioning Ca^{2+} channels and not to changes in their gating or permeability properties. The authors also suggested that hyperexcitability was due to the increased number of Ca^{2+} channels. When ethanol is withdrawn, the increased number of Ca^{2+} channels in hippocampal CA1 neurons gives rise to the hyperexcitability in ethanol withdrawal rats[80].

Effects of dihydropyridines in ethanol withdrawal. DHP Ca^{2+} channel antagonists decrease the hyperexcitability associated with withdrawal from chronic ethanol administration. They protect against both the tremor and convulsive behaviour that occur in mild withdrawal[76], and the spontaneous and audiogenic convulsions that follow chronic treatment with higher doses of ethanol[82]. DHP Ca^{2+} channel antagonists can also prevent adaptations to chronic ethanol treatment, if they are given chronically during the ethanol intake[76]. Such administration prevents the development of alcohol tolerance in animal models[17,78] by preventing the increase in the number of DHP-sensitive Ca^{2+} channels caused by the chronic ethanol treatment[17,74].

DHP Ca^{2+} channel antagonists have been shown to decrease the number of available DHP binding sites in peripheral tissues[83]. Two groups have investigated the effects of repeatedly administering DHPs together with ethanol[78,84]. In one series of experiments, an increase in available DHP binding sites in the brain was shown when ethanol alone is given, but this effect was prevented when a DHP Ca^{2+} channel antagonist was co-administered[85]. Both groups found that concurrent administration of the DHP significantly reduced the development of alcohol tolerance[78,84]. In subsequent experiments, administration of DHP Ca^{2+} channel antagonist with ethanol was shown to prevent AWS even when the DHP was withdrawn 24 hours before the removal of ethanol[77].

DHP Ca^{2+} channel antagonists protect against the effects of ethanol withdrawal. The questions that need to be asked are: whether the effects are produced by action at neuronal Ca^{2+} channels, and whether the action is selective for ethanol withdrawal. The sterospecificity of the isomers of the PN 200-110 Ca^{2+} channel antagonist in protecting against AWS was the same as that on Ca^{2+} channel conductance[76]. This stereospecificity, and the correlation with the dose required to displace a radiolabelled DHP from binding sites in the CNS, suggested that the effect was mediated by neuronal voltage-sensitive Ca^{2+} channels, rather than a non-specific action[76]. DHP Ca^{2+} channel antagonists were found to have weak anticonvulsant actions against some other forms of convulsions, such as pentylenetetrazol seizures, and no action against others, such as NMDA or electroshock seizures[86]. The evidence therefore suggested that the Ca^{2+} channel antagonists were selective for ethanol withdrawal signs, rather than that they possessed general anticonvulsant actions[86].

Littleton[81] and Dolin and Little[17] suggested that the increase in DHP binding sites after chronic administration of ethanol was the result of an adaptive up-regulation mechanism in the synthesis of Ca^{2+} channels. They put forward a theory to explain the effects of DHP Ca^{2+} antagonists on ethanol tolerance and withdrawal. The evidence (according to these authors) for this theory are: (1) acute ethanol decreases Ca^{2+} influx (conflicting data showing increased intracellular Ca^{2+} concentrations has been reported, 3.1.1), not necessarily through DHP-sensitive Ca^{2+} channels; (2) the number of DHP-sensitive Ca^{2+} channels in the CNS is increased after chronic ethanol treatment; and (3) concurrent chronic administration of DHP Ca^{2+} channel antagonists prevents the up-regulation of DHP binding, the development of ethanol tolerance and the behavioural and electrophysiological manifestations of the ethanol withdrawal syndrome.

Summary of ethanol–Ca^{2+} interactions

Both acute and chronic effects of ethanol on cellular Ca^{2+} have not shown any general consensus. However, there is strong evidence that chronic ethanol administration results in an increase in the number of DHP binding sites, which reflects the increase in DHP Ca^{2+} channels. In addition, it is suggested that ethanol physical dependence is associated with an adaptive up-regulation of DHP-sensitive Ca^{2+} channels on excitable cells. On removal of ethanol from a dependent animal, the increase in the number of Ca^{2+} channels on central neurons causes hyperexcitability expressed as AWS. Treatment with DHP Ca^{2+} channel antagonists can decrease the hyperexcitability, and prevent AWS. This agrees with the MLPI mechanism that acute ethanol treatment alters the neuronal membrane lipids, which then perturbs voltage-dependent Ca^{2+} channels. Subsequent adaptations in this system (up-regulation of DHP sensitive Ca^{2+} channels) occur after chronic administration of ethanol, and a sudden withdrawal of ethanol then causes hyperexcitation which results in AWS.

EFFECTS OF ETHANOL ON GABA RECEPTORS

GABA is a major inhibitory neurotransmitter in the CNS. It acts at two types of receptor: the $GABA_A$ site, at which it increases chloride (Cl^-) conductance and the $GABA_B$ site, coupled to adenyl cyclase which increases Ca^{2+} conductance. The $GABA_A$ receptor is part of a protein complex that also bears the benzodiazepine (BZD) receptor sites that bind picrotoxin and the barbiturates, and the Cl^- ionophore[87]. There appears to be at least two binding sites for GABA within this complex, each with different affinities. Most studies on the actions of ethanol on GABA transmission have involved the $GABA_A$ receptor ionophore.

The hypothesis that reduced inhibitory GABA-ergic action in the CNS might be responsible for hyperexcitability during ethanol withdrawal is based on two observations[96]. (1) Many clinical and animal studies showed that drugs known to augment GABA-ergic function can suppress AWS. For example, valproate and vigabatrin, which increase the availability of endogenous GABA, and agents like muscimol, barbiturates, and BZD, which interact more directly with GABA receptors, all suppress AWS. Conversely, drugs that blocked either GABA synthesis or GABA receptors can induce seizures that resemble AWS[97]. (2) Ethanol directly stimulates GABA-ergic neurotransmission. Although ethanol does not directly activate a specific receptor, some electrophysiological and biochemical results suggested that ethanol can either increase the efficiency of endogenous or exogenously applied GABA[97–100] or directly activate GABA-ergic receptor mechanisms via an allosteric interaction[99, 100].

Acute effects of ethanol on GABA/benzodiazepine (BZD) receptor complex

Acute administration of ethanol both *in vivo* and *in vitro* at relevant concentrations does not appear to alter GABA or BZD. Ticku[90], however, showed that acute ethanol treatment increased the density of GABA receptors. Tolerance to this effect occurred after prolonged ethanol intake. Davis and Ticku[91] showed that ethanol acted at the picrotoxin-sensitive site within the GABA–Cl^- ionophore to increase BDZ binding. This occurred at concentrations between 20 and 100 mM ethanol; above 100 mM ethanol there was less effect. An increase in the number and affinity of GABA receptors is expected to increase normal GABA transmission, but it is uncertain what effect the potentiation of BZD binding will have. Thyagarajan and Ticku[93] showed that at high concentrations (100–500 mM) ethanol non-competitively decreased the binding of *t*-butylbicyclophosphorothionate (TBPS), a ligand that binds to the picrotoxin site. Liljequist *et al*[94] also showed this effect of ethanol at 20–100 mM. Sanna *et al*[95] further demonstrated that this effect occurred after *in vivo* administration of low doses (0.5–1 g/kg) of ethanol and involved a decrease in the apparent affinity of the binding sites.

Electrophysiological studies. Various electrophysiological studies have shown potentiation of the actions

Table 1: *In vitro* GABA transmission (acute effects of ethanol).

Tissue (ref.)	Response study	[EtOH](mM)	Effects of Ethanol
Frog spinal cord (98)	Primary afferent depolarization	33–100	Increase in depolarization
Cultured mouse spinal cord cells (99)	Apply GABA	20–80	No change or small potentiation
Hippocampal slices CA1 pyrimidal cells (58)	Apply GABA	5–20 100	No potentiation Small potentiation
Hippocampal slices (100)	Inhibitory currents	10–200	No effect
Hippocampal slices CA1 and CA3 cells (60)	Apply GABA Inhibitory currents	22–88 10–350	No change Mostly decrease
Cultured chick spinal cord cells (102)	Apply GABA	20–50	Potentiation
Hippocampal slices CA1 pyrimidal cells (101)	Apply GABA	70	Potentiation
Superior colliculus	Apply ^{3}H-GABA	20–100	Decrease release
Substantia nigra (103)	Apply ^{3}H-GABA Apply ^{3}H-GABA	20–100 100–500	No change or increase release Inhibit release

of GABA *in vitro* with 33–100 mM of ethanol[101] as shown in Table 1. Gruol[102] found that cultured mouse spinal cord neurons showed either no change or a very small increase in the responses to applied GABA when 20–80 mM of ethanol was added. Carlen *et al*[61] using 5–20 mM ethanol concentrations, found no evidence of potentiation of GABA-ergic effects on CA1 pyramidal cells in isolated hippocampal slices, but some potentiation was seen with 100 mM ethanol. Gage and Robertson[103] found no detectable effects of ethanol (10–200 mM) on inhibitory synaptic currents in an isolated rat hippocampal slice.

No evidence of potentiation of GABA was seen by Siggins *et al*[63], who studied rat hippocampal slices using intracellular recording from CA1 pyramidal cells after application of GABA. Increases were not seen in the amplitude of the hyperpolarizing responses to GABA. In the majority of cells, ethanol (22–80 mM) either failed to alter or slightly reduced the GABA effect. Inhibitory postsynaptic potentials produced by stimulation of the stratum radiatum, thought to be mediated by GABA, were consistently reduced by ethanol, over the 10–350 mM concentration range. Takada *et al*[104] however, found that ethanol increased the inhibition by GABA of population spikes in CA1 cells (from hippocampal slices), when ethanol concentrations of 70 mM and higher were used. A voltage clamp study of chick spinal-cord neurons found that ethanol (20–50 mM) increased the actions of GABA[105]. In contrast, Gage and Robertson[103] found that bath-applied ethanol, at concentrations between 10 and 200 mM, had no effect on spontaneous GABA-mediated inhibitory postsynaptic currents, recorded by voltage clamp from pyramidal cells in hippocampal slices. Peris *et al*[106] noted that the effects of ethanol exposure *in vitro* on ^{3}H-GABA release differed depending on the region studied. Low concentrations (20–100 mM) decreased GABA release in the superior colliculus (SC), whereas the same concentrations of ethanol had no effect or increased release in the substantia nigra (SN). Only higher concentrations (100–500 mM) of ethanol inhibited release from SN.

The differences between these studies may have been due to the concentrations used, potentiation being somewhat more consistent with the higher ethanol concentrations, but this is clearly not the entire explanation for the disparate results. For instance, in one of the studies cited above, it was observed that the effect of ethanol was critically dependent on the duration of its application[105]. Short (10 second pulse) applications of ethanol, applied locally from a multibarrel pipette, caused consistent potentiation of the actions of applied GABA, while longer ethanol application (30 second pulses) did not have this effect. The authors suggested that the interaction between ethanol and GABA might involve multiple competing processes. This type of interaction may also account for some of the variability in the effects of ethanol on GABA inhibition.

Table 2: *In vivo* GABA transmission (acute effects of ethanol).

Tissue (ref.)	Response study	[EtOH](g/kg)	Effects of ethanol
Cerebral cortex (104)	Action potential frequency	Iontophoresis	Potentiation of effects of GABA
Cerebellar Purkinje cells	GABA-mediated inhibition	Iontophoresis	Decrease in inhibition
		1.5 (i.v.)	Decrease in inhibition
(107)	Action potential frequency	Iontophoresis	Decrease effects of GABA
Hoppocampus (105)	Action potential frequency	1.5	No consistent change in effects of GABA
Hippocampus (106)	Recurrent inhibition	2.0	Potentiation of inhibition

Even when studies *in vivo* are included, the picture is no clearer (Table 2). Nestoros[107] demonstrated selective potentiation of GABA-ergic actions by iontophoretically applied ethanol. Mancillas *et al*[108], however, found no consistent change in responses of hippocampal neurons to GABA after systemic administration of 1.5 g/kg ethanol. Wiesner *et al*[109] noted that i.p. injection of ethanol (2 g/kg) increased recurrent inhibition in the dentate gyrus, measured indirectly by pair-pulse stimulation. This may have been due to enhancement of GABA-mediated inhibition or by decreased pair-pulse potentiation, increased after hyperpolarization or decreased recovery of excitability. Harris and Sinclair[110], on the other hand, found that ethanol decreased GABA-mediated inhibition in cerebellar Purkinje cells.

There is a confounding consideration in these studies because the recordings were made under the influence of halothane anaesthesia. It is very difficult to interpret results obtained during the application of ethanol against a background of the actions of another general anaesthetic agent. The drug used to produce the anaesthesia may itself have already affected the actions of GABA, either directly or indirectly, and interactions may occur between the actions of ethanol and the other pharmacological agent.

Ethanol-enhanced increases in the action of GABA or GABA agonists on chloride (Cl^-) flux (measured biochemically) have been found consistently. Ticku and co-workers[100,111] using cultured spinal-cord neurons, found that ethanol increased the action of GABA over a 5–100 mM concentration range. Allan and Harris[98] showed that 10–45 mM ethanol, increased the action of muscimol on Cl^- uptake into membrane vesicles. Suzdak *et al*[112] using synaptoneurosomes, found increased GABA agonist action at an ethanol concentration of 10 mM. Ethanol was also found to have a direct action on synaptoneurosomes, increasing Cl^- uptake in the absence of GABA agonists. The ED_{50} for this was between 25 and 35 mM, while concentrations higher than about 60 mM were less effective[112].

The fact that the effects of ethanol on both GABA-induced Cl^- flux and GABA binding occurred over the concentration range which also produces behavioural effects suggests that these actions may be of importance in the mechanism of acute ethanol action. An increase in binding need not be necessary for potentiation of GABA actions. *In vitro* studies on Cl^- flux showed that potentiation occurred at concentrations produced by lower doses of ethanol than those needed to affect binding. This gives rise to the question, what causes the altered Cl^- flux if the effect is not through binding and why does it only occur at low ethanol concentrations.

The low concentrations that produce behavioural excitation (5–20 mM) have either no effect on GABA or qualitatively similar actions to the higher concentrations (50–100 mM). When the amount of ethanol is increased above the latter concentrations, the effects on GABA transmission either decrease or do not increase further. This is perhaps due to other actions of ethanol on the cell membrane that come into play at higher ethanol concentrations (toxic levels), preventing normal functioning of the GABA ionophore.

GABA release and reuptake. Effects of ethanol on GABA synthesis, release, and re-uptake are the subject of a number of earlier studies which yielded inconclusive results[97]. Ethanol (150–500 mM) inhibited K^+-stimulated release of GABA from cortical brain slices and synaptosomes[113] but a lower concentration (60 mM) was not effective[114]. An acute injection of ethanol did not change GABA content or GABA accumulation in the rat brain[115]. *In vitro* ethanol (up to 400 mM) did not alter synaptosomal high affinity GABA uptake[116]. Taken together, these observations suggest that non-toxic concentrations of ethanol has little effect on presynaptic regulation of GABA-ergic transmission.

Ethanol tolerance and dependence on GABA BZD receptor complex

Chronic ethanol administration has been found to decrease low-affinity GABA binding in a rodent brain[87,90] Rat studies by Ticku[90], showed that treatment with 11–17 g/kg/day ethanol, for 21 days, which

was sufficient to cause AWS, decreased the affinity of the low-affinity GABA binding site. This change was not seen after shorter treatment duration, which did not result in behavioural withdrawal, and was found only over the period during which audiogenic seizure activity was seen. Rastogi *et al*[117] studied the effects of chronic ethanol treatment on GABA stimulation of [^{35}S]-TBPS binding, a liquid that binds at the picrotoxin site, and GABA stimulation of [^{3}H]-flunitrazepam binding, and found that these interactions were unchanged. These allosteric effects were considered to reflect the coupling between GABA receptors and other sites on the GABA-Cl^- ionophore complex[117].

Hunt and Dalton[118] did not find changes in GABA binding while Harris *et al*[119] found GABA binding to be increased after chronic ethanol administration. The difference in results may be methodological. The latter study was carried out on brain tissue from miniature swine, and there may be species differences. Binding at the BZD receptor is either decreased[120, 121] or unchanged[122, 123] after chronic ethanol treatment. Deitrich *et al*[20] reported that GABA agonists and BZD agonists potentiated the effect of ethanol (potentiate GABA-ergic responses), whereas BZD antagonists blocked the potentiating effect of BZD agonists but did not affect the depressant effects of ethanol. These results suggested that ethanol can potentiate GABA-ergic effects but that it does not do so via a BZD-like action on the receptor-channel complex[20].

Allan and Harris[98] found that chronic ethanol treatment abolished the potentiation of muscimol-related Cl^- uptake into microsacs seen with acute ethanol (10–45 mM) (prepared from mouse cerebellar tissue). Morrow *et al*[124] demonstrated tolerance action of ethanol on Cl^- flux into synapneurosomes after chronic treatment with ethanol *in vivo* inhalation for 14 days. In the latter study, potentiation by ethanol of GABA-agonist stimulated Cl^- uptake was lost completely after chronic treatment. Takada *et al*[104] studied the effects of ethanol by extracellular recordings on hippocampal slices prepared from rats 4 days after cessation of chronic ethanol treatment (2 g/kg, twice daily for 30 days). These authors[104] found that inhibition by exogenous GABA resulted population spikes in the CA1 area of the hippocampal occurred to the same extent after chronic treatment as in control slices. The potentiation of this effect by ethanol at concentrations above 70 mM, seen in control preparations, was lost after chronic treatment. Whittington and Little[125] described the patterns of hyperexcitability that were seen in extracellular recording from isolated hippocampal slices, prepared immediately on withdrawal from chronic ethanol treatment. Their results suggested that several different mechanisms may underlie the withdrawal hyperexcitability, including a decrease in GABA-mediated inhibition produced by down regulation of GABA after chronic ethanol administration. In addition, chronic ethanol treatment has been reported to decrease the behavioural effects of GABA agonists, suggesting either down-regulation of GABA receptors or alterations in coupling[126, 127]. However, there are other studies that do not show behavioural adaptations to ethanol.

As mentioned before, GABA agonists, such as muscimol, BZD and THIP, and agents that increase GABA concentrations such as sodium valproate and vigabatrin, have been found to decrease AWS[128, 129]. BZD agonists have been suggested to be the drugs of choice in treating withdrawal in alcoholics, but they do not prevent the dependence, they merely decrease AWS[130]. In addition, BZDs are anticonvulsants effective against a wide variety of *in vitro* seizure models (other than those purely due to blockage of GABA transmission), including NMDA[131], digoxin[132], pilocarpine[133], and nicotine-induced seizures[134]. Therefore direct relevance to specific mechanism of AWS is still uncertain.

Chronic ethanol treatment has been reported to decrease the number and affinity of GABA receptor sites. Can these changes be responsible for the AWS? Careful and detailed studies by Ticku[90] showed that there was a temporal correlation between binding changes and the appearance of withdrawal behaviour. Decreases in GABA binding can be responsible for tolerance to those actions of ethanol that are produced by potentiation of GABA transmission. However, Harris *et al*[119] found no change in the number of GABA receptors after chronic ethanol intake. As mentioned before, the functional importance of the high affinity GABA site is uncertain, as it will be saturated at the normal GABA concentrations achieved in the synaptic cleft.

Summary of ethanol–GABA interactions

The hypothesis that ethanol potentiates GABA-ergic transmission is attractive and is quite consistent with many behavioural and neurochemical results. However, there are many electrophysiological observations that do not support this hypothesis. Attempting to resolve the apparent differences between the effects of ethanol on GABA-ergic responses in various preparations and laboratories may be premature, because currently available studies do not clearly show which experimental variables are important and which are not[20]. However, several hypotheses can be proposed that may prove important in integrating these findings. First, several studies indicate that only some components of the GABA response are affected. This suggests that with certain methods of

ethanol application, differences will not be observed because the transient component cannot be easily detected. Second, it is possible that the effect of ethanol is region specific within the brain.

Another difference may relate to recent findings that there are multiple forms of the subunits for the receptor[88, 135], which underlie the heterogeneity among the $GABA_A$ receptor subtypes. In addition, it is possible that these subunits may have different sensitivities to the effects of ethanol, whilst some subunit(s) might not even be affected by ethanol. For example, Wafford and Whiting[136] find that the γ2L subunit of the $GABA_A$ receptor is minimally present in hippocampal membranes. This then raises the possibility that the γ2L subunit may play an important role in AWS, whereas other subunits present in hippocampal membranes are not sensitive to ethanol. Further investigations on the homogeneous γ2L subunit will support this hypothesis. Unfortunately, no such investigation has been reported so far.

In general, brain regions in which GABA modulation by ethanol is typically not observed, such as the hippocampus, may have a receptor variant that is not sensitive to ethanol, whereas a receptor subtype that is affected will be expected in regions such as the cortex and possibly cerebellum, in which modulation is observed. This type of heterogeneity may exist even among cells from the same brain area. For example, using cultured spinal cord neurons, Study and Barker[137] found that, although all of the cells tested respond to GABA, the GABA response was potentiated by diazepam in 82% of the cells, by pentobarbital in 85% of the cells, and by both drugs in only 53% of the cells.

In summary, both behavioural and neurochemical results support the MLPI mechanism which acute administration of ethanol potentiates GABA-ergic transmission. After chronic administration of ethanol, subsequent adaptation by down regulating the GABA-ergic transmission occurs. A sudden withdrawal of ethanol then leads to hyperexcitation, and results in AWS.

EFFECTS OF ETHANOL ON GLUTAMATE RECEPTORS

Glutamate (Glu) is one of the primary neurotransmitters mediating excitatory neurotransmission in the CNS by means of ligand-gated cation channels[1]. Major excitatory neuronal systems in the brain, including the cortical pyramidal cells, primary sensory afferents, cerebellar granule cells, and the ascending excitatory pathways, use Glu or related excitatory amino acids as their neurotransmitter[138].

Glutamate receptors are widely distributed throughout the CNS. There are four subtypes of Glu receptors, designed as NMDA, kainate, α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA), and quisqualate[138]. These can be classified into two major families on the basis of their mechanisms of transduction: (1) ionotropic receptors, which are coupled to a cation ion channel, and (2) metabotropic receptors, which are coupled to inositol 1,4,5-triphosphate turnover mediated by G protein. The Glu-gated ionophores are the receptor family that show the best correlation with the diverse ethanol effects on the CNS. These ion channels can be further subclassified, according to their respective high affinity ligands, into NMDA, kainate, and AMPA subtypes.

The NMDA receptor is activated by Glu or NMDA, and channel opening mediates an increase in permeability to Na^+, K^+, and Ca^{2+}. The activity of the NMDA receptor complex is subjected to modulation by a number of agents, including Mg^{2+}, which causes a direct voltage-dependent blockage of the ion channel, Zn^{2+}, which decreases channel function in a voltage-independent manner at a site peripheral to the channel, and glycine (Gly), which modulates NMDA receptor activity allosterically through a strychnine-insensitive Gly recognition site and has been suggested as a co-agonist[139]. Within the channel there is also a site that binds phencyclidine and MK-801 to produce a non-competitive inhibition[140]. Additional modulation is produced by a site that mediates the NMDA-enhancing effects of the polyamines[141]. The NMDA receptors also play a major role in synaptic plasticity (e.g. long-term potentiation) and neurotoxicity (e.g. seizures and ischemic damage to neurons)[142]. It is the influx of Ca^{2+} which is believed to contribute to the involvement of the NMDA receptor to synaptic plasticity and development. However, when the receptor is excessively stimulated, the influx of Ca^{2+} can lead to seizure activity and cell death[141]. There is evidence that alcohol affects glutamatergic transmission in three ways: by interfering with fast excitatory neurotransmission, by promoting excitatoxicity (persistent or excessive activation of Glu receptors), and by impairing neurodevelopment[1].

Neurochemical effects of ethanol on glutamatergic transmission

During acute exposure, ethanol produces diverse effects on neurotransmission. Ethanol inhibits the release of serotonin, dopamine, norepinephrine, Glu, Asp, and GABA[143]. These effects are suggested to be indirectly mediated through the NMDA receptor[1]. Ethanol acts like an NMDA antagonist, inhibiting NMDA-stimulated Ca influx[144]. Gulya *et al*[145]

found that 100 mM of ethanol inhibited the non-equilibrium binding of [^{3}H]MK-801, which is an index of NMDA receptor-channel opening. This inhibition can be reversed in a time- and concentration-dependent manner by the addition of Gly. In addition, Gonzales and Woodward[146] found that the inhibition was also brain regional-dependent. In subsequent studies, Woodward *et al*[147], using rat striatal slices, showed that the ability of Gly to antagonize the ethanol-induced inhibition of NMDA-stimulated endogenous dopamine release was apparent at 25 mM, but not at ethanol concentrations of 50 mM and higher. This result suggested that even within brain regions, ethanol might possess multiple actions that cause a change in NMDA-stimulated processes.

Acute ethanol administration has no effect on Glu release, uptake, or tissue concentration in the forebrain. However, it causes a significant decrease in Asp and Glu concentrations in midbrain and brainstem, and decreases Glu tissue concentration (but not release) in the hippocampus[148], in agreement with the notion of regional brain dependence. Iorio *et al*[149] found that tolerance to some of these effects do not appear to develop, as there is no change in the ability of ethanol to inhibit the NMDA response in cerebellar granule cells after chronic exposure of neurons to ethanol.

When experimental animals were subjected to chronic ethanol administration, Michaelis *et al*[150] found that the number of Glu receptor binding sites increased in synaptosomal membranes. Gulya *et al*[4] and Grant *et al*[151] found that chronic ingestion of ethanol (7% ethanol for 7 days) produced increases in MK-801 binding sites by approximately 40–50%, a marker for NMDA receptors, in cerebral cortex, striatum, thalamus, and hippocampus, and that these increases lasted between 10 and 24 hours.

The increase in the number of MK-801 binding sites is probably a compensatory mechanism (up-regulation of NMDA receptors) for overcoming the ethanol-mediated inhibition of NMDA/Glu neurotransmission. This inhibitory effect of ethanol has been observed in cultured cerebellar granule cells[152] and hippocampal cells[11], as well as in slices of rat cortex[152] and striatum[153]. Thus, it is not surprising that NMDA receptors in these brain regions are up-regulated after chronic ethanol ingestion. However, it remains to be determined whether the increase in the number of MK-801 binding sites reflects an increase in function of NMDA receptors.

Several investigators have shown that both control and ethanol-treated cells are protected against neurotoxicity if the cells are exposed to Glu in the presence of high concentrations of the NMDA receptor antagonists CGS-19755[141] (a competitive antagonist of Glu), 5,7-dichlorokynurenate[141] (a Gly-site antagonist) or ADCI[151] (an ion channel antagonist). Snell *et al*[154] reported that chronic ethanol exposure did not affect the apparent affinities and densities of NMDA-specific [^{3}H]Glu, [^{3}H]CGS-19755, [^{3}H]Gly or [^{3}H]MK-801 binding sites. This result suggests that the inhibition of function is not mediated by direct competition of ethanol with ligand binding at any of these sites, which further suggests a specific modulation of ethanol on the NMDA channel ionophore instead. McCowan *et al*[148] found that intracellular Ca^{2+} concentration is significantly enhanced after chronic *in vitro* exposure to ethanol. The enhancement is consistent with an increased number of NMDA receptors, through which Ca^{2+} can enter neurons. The authors further reported that during chronic dependent states, Glu release is decreased; conversely, uptake and tissue concentration of Glu are increased. However, during the ethanol withdrawal state, Glu synaptic release, uptake, and tissue concentration are increased. Regional variations were observed, by Keller *et al*[155], with regard to these effects and the degree of effects.

In addition, Hoffman[170] reports that acute (2 hour, 200 μM) or chronic (3 days, 50 μM) exposure of cerebellar granule cells to ganglioside GM_1 protects control and ethanol-treated cells against Glu neurotoxicity. However, while the acute ganglioside GM_1 treatment does not interfere with initial response to Glu (increase in intracellular Ca^{2+}), this response is 'down-regulated' after chronic ganglioside GM_1 treatment. Furthermore, chronic ganglioside GM_1 treatment during ethanol exposure has the potential to prevent the ethanol-induced up-regulation of NMDA receptors that underlies AWS and the increased susceptibility to excitotoxicity, which further prevent withdrawal-induced neuronal damage[170].

Electrophysiological effects of ethanol on glutamatergic transmission

A predominant electrophysiological effect of ethanol is to reduce excitatory glutamatergic synaptic transmission[156]. Lovinger *et al*[8] found that ethanol inhibits the electrical current generated by NMDA receptor activation, and that this inhibition is concentration dependent. Concentrations of ethanol associated with intoxication *in vivo* correlate with those causing inhibition of NMDA currents. Simson *et al*[157] reported that ethanol produces a dose-dependent inhibition of the ability of NMDA to activate medial septal neurons *in vivo*. In addition, ethanol also potently inhibits NMDA-evoked neuronal activity in a current-dependent manner in the inferior colliculus and hippocampus. Clearly, the ability of ethanol to inhibit NMDA-evoked neuronal activity varies region-

ally. For example, Simson *et al*[158] found that ethanol fails to inhibit NMDA-evoked activity in the lateral septum. The complex regulatory responses of several NMDA subunits may explain the variation in the sensitivity of different neurons to ethanol as with GABA subunits to ethanol, or may be due to region-specific differences in inhibition. Molecular cloning of Glu receptor subunits and the functional expression of different combinations of Glu receptor subunits indicated that the NMDAR1 and NMDAR2C channels are slightly less sensitive to ethanol inhibition than the other heterometric channels[159]. Similarly, Lovinger[160] discovered that some forms of non-NMDA ionotropic Glu receptors have relatively high ethanol sensitivity, which appears to differ from that of endogenous AMPA or kainate receptors. In addition, Ishii *et al*[161] reported that the NMDAR1 mRNA is expressed throughout diverse brain regions, whereas NMDAR2 mRNA is distinctly distributed in different brain regions.

Pathophysiological studies

Lustig *et al*[162] demonstrated that acute ethanol administration protects against Glu-induced excitotoxic degeneration of cortical neurons. Acutely, neuroprotection by ethanol is accompanied by a decrease in the NMDA-evoked elevation of free intracellular Ca^{2+}. Because of its effect in attenuating glutamatergic transmission, acute ethanol administration protects against NMDA-induced convulsions. Also, MK-801 and ethanol have synergistic anticonvulsant effects[163]. However, chronic attenuation of Glu neurotransmission by ethanol results in compensatory up-regulation of NMDA receptors. As a consequence, ethanol withdrawal should be expected to be associated with increased excitatory neurotransmission. Consistent with this deduction, a non-selective Glu antagonist, glutamic acid diethyl ester, attenuates the seizures associated with withdrawal of ethanol after chronic ingestion[164]. Several investigators[154, 163] found that NMDA exacerbates, while MK-801 and other NMDA receptor antagonists decrease, the occurrence and severity of AWS. During withdrawal, Valverius *et al*[165] reported that mice have 50–70% more MK-801 binding sites. Ethanol-naive mice prone to withdrawal seizures have been found to have more MK-801 binding sites in the hippocampus.

Chronic exposure to ethanol can sensitize neurons to the excitotoxic effect of NMDA receptor activation because of the up-regulation of the NMDA receptors[166, 167]. Of 11 studies addressing potential alterations in NMDA-receptor-mediated neurotransmission with ethanol dependence, 10 studies of rodents indicated that long-term exposure to ethanol causes up-regulation of NMDA receptor density or its Ca^{2+}-mediated events[149, 151, 154, 165–167].

Excitatory amino acids, including Glu and Asp, exert trophic influences on neuronal differentiation and are thus important for neurodevelopment[168]. They regulate the formation of neuronal circuitry and synaptic plasticity. Activation of NMDA receptors enhances synaptogenesis in the hippocampus[169]. Glu exposure accelerates the maturation of synaptic profiles. Exposure to excitatory amino-acid receptor antagonists may disrupt the maturational processes mediated by excitatory amino acids and interfere with normal neurodevelopment.

Summary of ethanol–glutamate interactions

Overall, the results indicate a significant role for increased NMDA receptor function in both AWS and in the neuronal damage that is associated with chronic ethanol exposure and withdrawal. The increase in NMDA receptor function observed in ethanol-treated animals and cells may represent an adaptive response to the initial inhibition of NMDA receptor function by ethanol. The presence of ethanol in the cells is expected to protect the cells from the consequences of NMDA receptor up-regulation, which will appear after chronic treatment of ethanol, and result in AWS after ethanol is eliminated or removed. This further supports the MLPI mechanism in which acute ethanol treatment alters neuronal membrane lipids, resulting in an inhibition of glutamatergic transmission. Subsequent adaptation of this system (by up-regulation of NMDA receptors) occur after chronic treatment of ethanol. A sudden withdrawal of ethanol then causes hyperexcitation which leads to AWS.

As for neuronal damage, it is likely that the repeated up-regulation of NMDA receptors is a key factor. However, the question of how acute ethanol exposure results in decreased NMDA-stimulated Ca^{2+} influx which leads to an adaptive response still remains unanswered. Various reports support the contention that ethanol withdrawal hyperexcitability can be treated with drugs that are antagonists at the NMDA receptor. In addition, chronic ganglioside GM_1 treatment during ethanol exposure has the potential to prevent the ethanol-induced up-regulation of NMDA receptors and thus AWS, which further prevents withdrawal-induced neuronal damage.

CONCLUSION

Ethanol produces alterations in neuronal membrane function, ranging from the activities of membrane-bound enzymes to the activities of ion channels and

neurotransmitter receptors. However, the extent to which these alterations affect membrane properties remains incompletely elucidated. More recently, it has been noted that certain membrane-bound proteins, such as $GABA_A$ receptors and NMDA receptors, are affected by low concentrations of ethanol (less than 50 mM), whereas other membrane-bound proteins, such as voltage-dependent Ca^{2+} channels, are affected only at higher concentrations of ethanol. These differential responses to low and high concentrations of ethanol do not necessarily preclude membrane fluidization as the underlying mechanism. The bilayers of biomembranes consist of heterogeneous types of phospholipids, and different phospholipids have different sensitivities to the fluidizing effects of ethanol. Therefore, it is possible that the responsiveness of a membrane-bound protein to ethanol depends upon the type of lipids present in the membrane microenvironment.

Studies have shown that membrane fluidity is unlikely to affect the activity of certain membrane-bound proteins directly, in particular those that are subjected to complex biochemical regulation, such as receptor gated ion channels. Rather, the alteration of membrane fluidity may perturb regulatory events that in turn affect the activity of these membrane-bound proteins or the membrane surface potential. These effects can then affect the $GABA_A$ receptors, NMDA receptors and/or voltage-dependent Ca^{2+} channels synergistically or in any combination. The effects include an increase of $GABA_A$ function, and a decrease of both NMDA action and voltage-dependent Ca^{2+} channel function, which result in different changes depending on brain region and/or cellular ethanol sensitivity. Subsequent adaptation of these cells, results in increased NMDA receptors and voltage-dependent Ca channels (up-regulation), and decreased $GABA_A$ function (down- regulation). A sudden withdrawal of ethanol then causes hyperexcitation which results in AWS. This hypothesis (MLPI mechanism) explains why there is not a strong consensus for a single mechanism among the enormous number of studies that have been performed; none the less, these studies do show that each mechanism plays an important role in AWS.

Nevertheless, this hypothesis, as well as all the studies that have been presented in this review, do not explain how membrane fluidity actually causes changes in GABA and NMDA receptors, and calcium channels. As previously noted, ethanol has the potential to disrupt the surface membrane potential. This then raises questions: Can the alteration of surface membrane potential affect ion channels (i.e. ion flux)? How significant will changes of ion flux affect the activity of these membrane-bound proteins? Is the alteration ion channel specific? To answer these questions, further experimental investigations are required, such as studies on surface membrane potentials with voltage-dependent Ca^{2+} flux current, $GABA_A$-activated Cl^- current and ion current activated by NMDA.

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